

## LIVE GENETICALLY ATTENUATED MALARIA VACCINE

## CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application  
5 No. 60/631,228, filed November 26, 2004, and U.S. Provisional Application  
No. 60/531,479, filed December 19, 2003, all three of which are incorporated by  
reference in their entireties.

## STATEMENT OF GOVERNMENT LICENSE RIGHTS

The U.S. Government has a paid-up license in this invention and the right in  
10 limited circumstances to require the patent owner to license others on reasonable terms as  
provided for by the terms of RO1 A053709 awarded by the National Institutes of Health.

## FIELD OF THE INVENTION

This invention relates to live genetically modified *Plasmodium* organisms and  
their use as immunospecific immunoeffectors for vaccination purposes.

## 15 BACKGROUND OF THE INVENTION

Malaria has a tremendous impact on human health, killing millions annually and  
the disease is a major impediment for social and economic development of nations in  
malaria-endemic areas, particularly in sub-Saharan Africa (1, see the appended Citations).  
Malaria is a mosquito-borne disease that is transmitted by inoculation of the *Plasmodium*  
20 parasite sporozoite stage. Sporozoites invade hepatocytes (2), transform into liver stages,  
and subsequent liver stage development ultimately results in release of pathogenic  
merozoites (3).

Because an effective 'subunit' malaria vaccine has remained elusive and the  
complexity of the malaria parasite *Plasmodium* might preclude the successful  
25 development of such a vaccine, whole organism vaccine approaches against malaria have  
lately found renewed interest (4). The feasibility of such a vaccine has been  
demonstrated in animal models and subsequently in humans by induction of sterile  
protective immunity through inoculation with irradiation-attenuated parasites (5, 6).  
Liver stages are a prime malaria vaccine target because they can be completely  
30 eliminated by sterilizing immune responses, thereby preventing malaria infection (7).  
The recent availability of complete *Plasmodium* genome sequences (8, 9) may now  
permit the development of live-attenuated parasites by more precise and defined genetic  
manipulations.

Using expression profiling, we previously identified genes that are specifically expressed during the pre-erythrocytic part of the parasite life cycle (11, 12). A number of pre-erythrocytic genes named *UIS* (up-regulated in infective sporozoites) also showed up-regulation in sporozoites when they gain infectivity for the mammalian host (11).

## SUMMARY OF THE INVENTION

Here we show by reverse genetics that selected individual genes, exemplified by *UIS3* (up-regulated in infective sporozoites gene 3) and *UIS4*, are essential for early liver stage development: *uis3*(-) and *uis4*(-) sporozoites infect hepatocytes but are no longer able to establish blood stage infections *in vivo* and thus do not lead to disease. The invention thereby provides the first live *Plasmodium* organisms that are genetically engineered to disrupt liver-stage-specific gene functions

Surprisingly, immunization with either *uis3*(-) or *uis4*(-) sporozoites confers complete protection against infectious sporozoite challenge in a rodent malaria model. This protection is sustained and stage-specific. These findings provide the first genetically attenuated whole organism malaria vaccines.

Thus, the invention provides a method for inoculating a vertebrate host against malaria, by administering to the host a live *Plasmodium* organism that is genetically engineered to disrupt a liver-stage-specific gene function. The invention further provides a vaccine composition comprising a live *Plasmodium* organism that is genetically engineered to disrupt a liver-stage-specific gene function. In addition, the invention provides the use of a vaccine composition comprising a live *Plasmodium* organism that is genetically engineered to disrupt a liver-stage-specific gene function. The invention also provides for production of a vaccine composition, by suspending the subject engineered *Plasmodium* organisms in a suitable pharmaceutically acceptable carrier solution.

## BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1 depicts the primary structure of *Plasmodium* *UIS3* proteins, as described in Example 1; and

FIGURE 2 depicts the replacement strategy used to generate the *uis3(-)* parasite described in Example 1.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The invention provides a method for inoculating a vertebrate host against a *Plasmodium* parasite, by administering to the host a live *Plasmodium* organism that is genetically engineered to disrupt a liver-stage-specific gene function.

By *Plasmodium* parasite is meant any member of the protozoan genus *Plasmodium*, including the four species that cause human malaria: *P. vivax*, *P. malariae*, *P. falciparum*, and *P. ovale*. The corresponding vertebrate host is a human or other secondary host that is susceptible to infection by the wild-type *Plasmodium* parasite.

For use as a live anti-malarial vaccine, the *Plasmodium* parasite is genetically engineered to disrupt a liver-stage-specific gene function. The term "disrupt liver-stage-specific gene function" or "disrupt LS-specific gene function" means interfering with an LS-specific gene function such as to completely or partially inhibit, inactivate, attenuate, or block the LS-specific gene function, for example, by gene disruption or influencing transcription, translation, protein folding, and/or protein activity. The term "liver-stage-specific gene function" or "LS-specific gene function" refers to a function that is required in liver stage parasites to ultimately produce infectious merozoites and establish the erythrocytic stage of the life cycle, but that is not required for entry into host hepatocytes or maintenance of the parasite in asexual blood cell stages and production of infective sporozoites. Malaria infection is initiated by *Plasmodium* sporozoites in the salivary glands of mosquitoes. These sporozoites invade hepatocytes of the vertebrate host and differentiate into liver stage (LS) forms. After a few days the LS parasites produce several thousand merozoites that are released from the hepatocytes and invade erythrocytes to start the blood stage cycle that causes malaria disease. According to the invention, the *Plasmodium* parasite is genetically engineered to disrupt at least one LS-specific gene function such that the genetically engineered parasites remain capable of invading hepatocytes but cannot produce merozoites that can establish blood stage infections.

An LS-specific gene function may be identified using routine methodology that is standard in the art. For example, an LS-specific gene function may be identified by assessing the function of genes whose expression is up-regulated in liver-stage parasites (LS-up-regulated genes). For example, genes whose expression is up-regulated in liver-

stage parasites may be expressed at higher levels in liver-stage parasites than in the sporozoite population that emerges from mosquito mid-gut oocysts. Up-regulation of expression of such genes may also be observed in mature, infective salivary gland sporozoites (like in the *UIS4* and *UIS3* genes discussed in the Examples below). Well-known methods for differential transcriptional profiling, including, but not limited to, subtractive hybridization screens, differential display, and genome-wide microarray analyses, may be used for identifying genes whose expression is up-regulated in liver-stage parasites. Such methods have been previously used to analyze infectivity-associated changes in the transcriptional repertoire of sporozoite-stage parasites (11) and to identify *Plasmodium* genes that encode pre-erythrocytic stage-specific proteins (12). For example, suppression subtractive hybridization permits selective enrichment of differentially regulated cDNAs of high and low abundance through a combination of hybridization and polymerase chain reaction (PCR) amplification protocols that allow the simultaneous normalization and subtraction of the cDNA populations. Suppression subtractive hybridization has been used to analyze transcriptional differences between non-infective and infective sporozoites and to identify genes controlling infectivity to the mammalian host (11). This procedure has permitted the identification of LS-up-regulated genes, including, but not limited to, *UIS3* and *UIS4*, as further described in the Examples below. Suppression subtractive hybridization of *Plasmodium* salivary gland sporozoites versus merozoites has also been used to identify stage-specific pre-erythrocytic transcripts (12). Differential expression of candidate LS-specific genes may be confirmed using methods that are standard in the art, including dot blots, reverse transcriptase PCR (RT-PCR), immunoblotting, immunofluorescence microscopy, and/or microarray expression analyses, as previously described (11, 12).

In some embodiments of the invention, LS-specific gene functions are identified by analyzing the function of LS-up-regulated genes, as further described below. However, not all genes with an LS-specific gene function are necessarily LS-up-regulated genes. Thus, genes whose expression is not up-regulated in LS forms may nevertheless possess an LS-specific gene function.

Interference with a liver-specific function may also be achieved by LS-specific overexpression of an inhibitory factor. This factor may be inserted by reverse genetics methods into a pseudogene, i.e., one that is not essential for parasite survival at any time

point during the life cycle (47). The inhibitory factor should not confer toxicity to the parasite but rather act in arresting LS development. Such a factor may include, but is not limited to, inhibitors of cell-cycle progression and/or ubiquitin-mediated proteolysis, and/or factors that interfere with post-transcriptional control of gene-expression.

5 LS-specific gene functions may be identified by analyzing the phenotype of parasites in which one or more gene functions have been disrupted. Several methods for disrupting gene functions in *Plasmodium* are well-known in the art and may be used in the practice of the invention. Such methods include, but are not limited to, gene replacement by homologous recombination, antisense technologies, and RNA  
10 interference. For example, methods of gene targeting for inactivation or modification of a *Plasmodium* gene by homologous recombination have been previously described (13). Such methods are herein successfully used to disrupt LS-specific gene functions, as described in Examples 1 and 2. Antisense technology has also been successfully used for disrupting *Plasmodium* gene functions. For example, exogenous delivery of  
15 phosphorothioate antisense oligonucleotides against different regions of the *P. falciparum* topoisomerase II gene result in sequence-specific inhibition of parasite growth (14). Similarly, transfection of an antisense construct to the *Plasmodium falciparum* clag9 gene, which had been shown to be essential for cytoadherence by targeted gene disruption, resulted in a 15-fold reduction in cytoadherence compared to untransfected  
20 control parasites (15).

Another exemplary technology that may be used in the practice of the invention to disrupt LS-specific gene functions is RNA interference (RNAi) using short interfering RNA molecules (siRNA) to produce phenotypic mutations in genes. RNAi has been used as a method to investigate and/or validate gene function in various organisms, including  
25 plants, *Drosophila*, mosquitoes, mice, and *Plasmodium* (see, e.g., 37-44). In *Plasmodium*, RNAi has been used, for example, to demonstrate the essential role of a PPI serine/threonine protein phosphatase (PfPP1) from *P. falciparum* (41). RNAi has also been used to inhibit *P. falciparum* growth by decreasing the level of expression of the gene encoding dihydroorotate dehydrogenase (42) and by blocking the expression of  
30 cysteine protease genes (43). In the mouse malaria model, RNAi has been used to inhibit gene expression in circulating *P. berghei* parasites *in vivo* (44). These studies have

demonstrated the use of RNAi as an effective tool for disrupting gene function in *Plasmodium* organisms.

The gene disruption approaches described above (for example, gene targeting by homologous recombination, antisense, and RNAi) have been used successfully to investigate the function of virtually all genes in an organism's genome. For example, the availability of sequenced genomes has enabled the generation of siRNA libraries for use in large-scale RNAi studies to screen for genes that are involved in various processes, such as developmental pathways or stages (see, e.g., 45 and 46). Such screens may be used in the practice of the invention to identify LS-specific gene functions in *Plasmodium*. Assays that may be used for identifying LS-specific gene functions include, but are not limited to, phenotypic analyses such as the phenotypic assays described in Examples 1 and 2. The term 'phenotypic analysis' includes all assays with vital recombinant parasites that are generated in a wild type, fluorescent or any other transgenic reporter background. Assays may be performed *in vivo*, with cultured cells, in *in vitro* development assays or any other system that provides a read-out for LS development.

The engineered *Plasmodium* organisms in which an LS-specific gene function has been disrupted are typically grown in cell culture or animals, expanded in the mosquito host, and harvested as sporozoites for use in vaccines (see, e.g., 16).

The subject vaccine compositions are produced by suspending the attenuated live *Plasmodium* organisms in a pharmaceutically acceptable carrier. Suitable pharmaceutically acceptable carriers include sterile water or sterile physiological salt solution, particularly phosphate buffered saline (PBS), as well known in the art.

Vaccines according to the invention can be administered, e.g., intradermally, subcutaneously, intramuscularly, intraperitoneally, and intravenously.

Dosage is empirically selected to achieve the desired immune response in the host. By immune response is meant an acquired and enhanced degree of protective immunity, preferably complete or sterile protection, against subsequent exposure to wild-type *Plasmodium* sporozoites. In the working examples described below, sterile protection was achieved following three vaccinations with 10,000 live genetically attenuated sporozoites per inoculation.

## DETAILED TECHNICAL DESCRIPTION

Background. Radiation-attenuated sporozoites are a singular model that achieves sterile, protective immunity against malaria infection.

Malaria causes more than 300 million clinical cases and more than 1 million death annually. The disease has a severe negative impact on the social and economic progress of developing nations. Transmission of the malaria parasite *Plasmodium* to the mammalian host occurs when infected mosquitoes bloodfeed and inoculate the sporozoite stage (spz). After entering the bloodstream, spzs are quickly transported to the liver where they extravasate and invade hepatocytes (2). Within hepatocytes, spzs transform into liver stages (LS) (also called exo-erythrocytic forms, EEFs). LS parasites grow, undergo multiple rounds of nuclear division and finally produce thousands of merozoite (17, 18). Merozoites released from the liver rapidly invade red blood cells and initiate the erythrocytic cycle, which causes malaria disease. A protective malaria vaccine would have tremendous impact on global health but despite over a century of efforts, no vaccine has been developed that confers prolonged protection. Yet, we have known for more than 35 years that sterile protracted protection against malaria infection is possible.

Immunization of mice with radiation-attenuated rodent model malaria spzs (gamma-spzs) induces sterile immunity against subsequent infectious spz challenge, thus completely preventing the initiation of blood stage infection from the liver (5). Importantly, based on these findings it was later shown that immunization of humans with gamma-*P. falciparum* spzs completely protected greater than 93% of human recipients (13 of 14) against infectious spz challenge and that protection can last for at least 10 months (6). Gamma-spzs retain the capacity to infect the liver of the mammalian host and invade hepatocytes (19-20). However, LS derived from gamma-spzs suffer arrested development and thus do not produce red blood cell-infectious merozoites. Although, the inoculated stage is the spz, the main immune target is the infected hepatocyte harboring the LS (21). Protective immunity is spz dose and radiation dose dependent: greater than 1000 immunizing bites from *P. falciparum*-infected mosquitoes exposed to 15,000–20,000 rads of gamma radiation is required to protect the majority of subjects exposed to infectious spz challenge (6). Mosquitoes inoculate between 10-100 spzs during a bite (22-23). Therefore, the total spz dose for complete protection comes to 10,000-100,000. Importantly, immunization with over-irradiated spzs or heat-

inactivated spzs fails to induce protection, indicating that the spz must remain viable for some time after inoculation and must progress to a liver stage that induces protection (6, 24). On the basis of observations in the rodent malaria model, protracted protective immunity may depend on sufficient expression of LS antigen (Ag), because treatment with primaquine, a drug that kills LS, aborts the development of protection (21). Importantly, protection induced by *P. falciparum* gamma-spzs is strain-transcending: inoculation with gamma-spzs of one parasite strain confers protection against heterologous strains (6).

Although we have learned much about spz gene expression in the last few years (25-27) the LS as the true immunological target of gamma-spzs induced protection have so far completely eluded gene expression analysis because of their inherent experimental inaccessibility. We currently know only one liver stage-specific Ag, liver stage antigen-1 (LSA-1) (28). Thus, the fine Ag specificity of lymphocytes participating in protective immunity remains unknown in humans, because the Ags expressed by LS parasites remain unknown.

Feasibility to create genetically attenuated *Plasmodium* Liver Stages. To generate genetically attenuated *Plasmodium* LS that are defective only in LS development a stage-specific gene that plays an essential and exclusive role at this stage needs to be disrupted. The gene cannot be essential during the blood stage cycle given that *Plasmodium* is haploid and transfection is done with asexual blood stages and the mutant parasites are maintained as blood stages (13). We previously employed transcription-profiling based on the prediction that infectious *Plasmodium* spzs residing in the mosquito salivary glands are uniquely equipped with transcripts required for hepatocyte invasion and subsequent development of the LS (11). Next, we screened for transcripts that are specific for pre-erythrocytic and absent from blood cell stages in order to generate a subset of genes that can be disrupted (12). The combined screens identified two abundant salivary gland spz enriched transcripts that are absent from blood stages, termed *UIS3* and *UIS4* (for uprergulated in infectios spzs). Cell biological studies showed that both encoded proteins locate to the parasitophorous vacuole, the parasite-derived organelle where replication and schizogony takes place (data not shown).

Gene knockouts using insertion and replacement strategies have now revealed that both genes are necessary for LS development (see Examples 1 and 2 below). Both proteins are already expressed in spzs (data not shown) but *uis3*(-) and *uis4*(-) parasites



develop normal spzs and these invade hepatocyte normally. However, *uis3*(-) and *uis4*(-) LS arrest in intermediate-LS development and do not produce late LS (data not shown). Therefore, both *UIS3* and *UIS4* have LS-specific gene functions. Importantly, animals infected by natural bite or intravenously with doses of up to 10,000 spzs do not become patent, confirming that both genes play vital roles in successful completion of the *Plasmodium* life cycle (see Tables 1 and 2 below). Therefore, we succeeded in generating the first genetically attenuated LS. Based on these discoveries we and others can now advance and test various LS-up-regulated genes identified by microarray analysis for their importance in LS development. We predict that more LS-up-regulated genes will turn out to be essential for LS development (i.e., to possess LS-specific gene functions), especially uniquely expressed genes given the remarkable capacity of the parasite to develop from a single spz to more than 10,000 daughter merozoites. Such LS-up-regulated genes can be similarly disrupted to produce additional live vaccine candidates, as described herein.

Representative embodiments of the present invention are described in the following two working examples.

#### EXAMPLE 1

This first Example was published by *Nature* AOP on December 5, 2004 (29).

We hypothesized that inactivation of *UIS* genes for which expression is restricted to pre-erythrocytic stages could lead to attenuation of the liver stage parasite, without affecting the blood stages or mosquito stages. We focused on a gene called *UIS3* that encodes a small conserved transmembrane protein (FIGURE 1). *UIS3* was expressed in infectious sporozoites (12) and we determined that it was also expressed after sporozoite infection of livers *in vivo* (data not shown). *UIS3* of rodent malaria parasites (accession number EAA22537) and *UIS3* of the human malaria parasite *P. falciparum* (Pf13\_0012) show 34% amino acid sequence identity (FIGURE 1). Because the rodent malaria parasites such as *P. berghei* (Pb) are excellent models to study *Plasmodium* liver stage and pre-erythrocytic immunity we pursued investigation of *UIS3* in this species.

The endogenous *PbUIS3* gene was deleted using a replacement strategy (13) (FIGURE 2). After transfection, parental blood stage parasites were used to obtain clonal parasite lines designated *uis3*(-) that contained exclusively the predicted locus deletion (data not shown). As expected, *uis3*(-) parasites showed normal asexual blood stage

growth and normal transmission to the *Anopheles* mosquito vector (data not shown). Within the mosquito *uis3*(-) sporozoites developed normally in midgut oocysts and infected the salivary glands in numbers comparable to wildtype (WT) sporozoites (data not shown). Reverse transcriptase (RT)-PCR confirmed lack of *UIS3* expression in *uis3*(-) sporozoites (data not shown). *uis3*(-) sporozoites showed typical gliding motility, a form of substrate-dependant locomotion that is critical for sporozoite transmission and infectivity (30) (data not shown). They also retained their host cell invasion capacity of cultured hepatoma cells at levels comparable to WT parasites (data not shown).

Intracellular *uis3*(-) sporozoites initiated the typical cellular transformation process that leads to de-differentiation of the banana-shaped elongated sporozoite to a spherical liver trophozoite(17, 31) (data not shown). In marked contrast, *uis3*(-) parasites showed a severe defect in their ability to complete transformation into liver trophozoites (data not shown). Only a small fraction of *uis3*(-) parasites developed into spherical early liver stages that also appeared consistently smaller than the corresponding WT forms. Consequently, mutant parasites lacked the capacity to progress to mature liver schizonts (data not shown). Based on this extreme developmental defect observed *in vitro*, we next tested if *uis3*(-) sporozoites had lost their capacity to progress through liver stage development and lead to blood stage infections *in vivo*. Indeed, intravenous injection of up to 100,000 *uis3*(-) sporozoites failed to induce blood stage parasitemia in young Sprague/Dawley rats which are highly susceptible to *P. berghei* sporozoite infections (data not shown). Control WT sporozoites induced blood stage parasitemia in rats between 3-4 days after injection.

Thus, the observed phenotypic characteristics of *uis3*(-) parasites, i.e., their ability to invade hepatocytes and their defect in complete liver stage development allowed us to test them as a whole organism vaccine in a mouse/sporozoite challenge model. We intravenously immunized mice with *uis3*(-) sporozoites using different prime-boost regimens and subsequently challenged the mice by intravenous injection of infectious WT sporozoites (Table 1). Protection was evaluated by blood smear to detect the development of blood stage parasitemia starting two days after sporozoite challenge, the most stringent readout for sterile protection against malaria infection. Priming with 50,000 *uis3*(-) sporozoites followed by 2 boosts with 25,000 *uis3*(-) sporozoites completely protected all immunized mice against a challenge with 10,000 WT sporozoites given 7 days after the last boost (Table 1). Complete sterile protection

against the same sporozoite challenge dose was also achieved with a similar prime-2 boost protocol using 10,000 *uis3*(-) sporozoites (Table 1). We next immunized mice using the same prime-boost protocols but challenged with WT sporozoites 4 weeks after the last boost. None of the challenged mice developed blood stage infections and thus enjoyed protracted sterile protection (Table 1). Protracted protection was confirmed by a re-challenge experiment where protected animals were challenged again with a high inoculum of 50,000 infectious sporozoites after two months. All animals remained completely protected. Mice immunized with *uis3*(-) sporozoites were also completely protected against re-challenge by infectious mosquito bite (Table 1). To determine the level of protection with a reduced immunization dose we tested a prime-single boost protocol with 10,000 *uis3*(-) sporozoites. Seven out of ten animals enjoyed complete protection, while the remaining three animals became patent after a long delay in patency. Next, a subset of immunized mice was challenged by direct inoculation with blood stage parasites. All animals developed blood stage parasitemia two days after challenge, indicating that the observed protective immunity is not acting against blood stages and thus was specific against pre-erythrocytic stages. Finally, to evaluate a more vaccine-relevant delivery route we immunized mice subcutaneously using a prime-2 boost protocol with 50,000 *uis3*(-) and 25,000 *uis3*(-) sporozoites, respectively. All mice were completely protected against subsequent intravenous WT sporozoite challenge.

Our results show that it is possible to develop genetically modified malaria parasites that are completely attenuated at the liver stage, which normally establishes infection of the mammalian host after mosquito transmission. This attenuation was achieved by deletion of a single parasite gene, *UIS3*. Although *UIS3* function remains unknown, *uis3*(-) parasites clearly lacked the ability to compensate for its loss. The protracted sterile protection against malaria that we observed after immunization with *uis3*(-) sporozoites in the mouse/sporozoite challenge model provides proof of principle that a genetically modified malaria vaccine is feasible. We identified a *UIS3* orthologue (accession number PF13\_0012) in the genome of the most lethal human malaria parasite *P. falciparum*. This will allow us to create a genetically attenuated *uis3*(-) human parasite that can be tested as a vaccine in human/sporozoite challenge models. Together our findings lead the way to the development of a genetically attenuated, protective whole organism malaria vaccine that prevents natural infection by mosquito bite.

Methods: *Plasmodium berghei* transfection. For replacement of *PbUIS3* two fragments were amplified using primers: UIS3rep1for (5' GGGTACCCGCATTAGCATAACATCTCATTGG 3') (SEQ ID NO: 1) and UIS3rep2rev (5' CAAGCTTGCTTTCATATATTTGTTATTTGTC 3') (SEQ ID NO: 2) for the 800 bp 3' fragment; and: UIS3rep3for (5' GGAATTCCCATATGTTTGTGTAACATC 3') (SEQ ID NO: 3) and UIS3rep4rev (5' CTCTAGAGTGTGCTTAAATGTTTCTTTAAAC 3') (SEQ ID NO: 4) for the 760 bp 5' fragment using *P. berghei* genomic DNA as template. Cloning into the *P. berghei* targeting vector (13) resulted in plasmid pAKM19. To obtain clonal parasite populations, limited dilution series and i.v. injection of one parasite into 15 recipient NMRI mice each was performed. For RT-PCR analysis we dissected  $6 \times 10^5$  *uis3*(-) and  $6 \times 10^5$  WT salivary gland sporozoites and isolated polyA<sup>+</sup> RNA using oligo dT-columns (Invitrogen). For cDNA-synthesis and amplification we performed a two step-PCR using random decamer primers (Ambion) and subsequent standard PCR reactions.

Phenotypical analysis of *uis3*(-) parasites. *Anopheles stephensi* mosquito rearing and maintenance were under a 14 h light/10 h dark cycle, 75% humidity and at 28°C or 20°C, respectively. For each experiment, mosquitoes were allowed to blood-feed for 15 min. on anaesthetized NMRI-mice that had been infected with wild-type *P. berghei* NK65 or the *uis3*(-) clone and were assayed for a high proportion of differentiated gametocytes and microgametocyte-stage parasites capable of exflagellation. Mosquitoes were dissected at days 10, 14, and 17 to determine infectivity, midgut sporozoite and salivary gland sporozoite numbers, respectively. For analysis of sporozoite motility, sporozoites were deposited onto precoated (3% BSA/RPMI 1640) glass coverslips, fixed for 10 min at RT with 4% paraformaldehyde, and incubated using primary antibody against *P. berghei* circumsporozoite protein (anti-PbCSP) (32). To detect liver stages in hepatocytes,  $10^3$  Huh7 cells were seeded in eight chamber slides and grown to semiconfluency. *P. berghei* sporozoites were added, incubated 90 min. at 37°C, and washed off. After 8, 12, 15, 24, 36 and 48 h, LS were revealed using primary antibodies against the *P. berghei* heat shock protein 70 (HSP70) (33). To analyze sporozoite invasion a double staining protocol with anti-CSP antibody was used (34). To determine the infectivity of clonal sporozoite populations *in vivo* young Sprague-Dawley rats were injected intravenously with 100 microliter sporozoite suspension in RPMI 1640.

Parasitemia of the animals was checked daily by Giemsa-stained blood smears. The appearance of a single erythrocytic stage represents the first day of patency.

Immunization and parasite challenge experiments. For all experiments female C57BL/6 mice (Charles River Laboratories) at the age of 50 to 80 days were used. For immunization, *uis3*(-) sporozoites were extracted from salivary glands from infected mosquitoes. Typically, a single infected mosquito contained 20,000 *uis3*(-) sporozoites. Sporozoites were injected in a volume of 100 microliters intravenously into the tail vein or subcutaneously into the neck of animals. Animals were immunized with a single dose of 1 or  $5 \times 10^4$  *uis3*(-) sporozoites, followed by two boosts of either 1 or  $2.5 \times 10^4$  *uis3*(-) sporozoites administered i.v. or s.c. The first boost was given 14 days following the immunization, with a second boost following 7 days thereafter, or at time intervals indicated. One set of animals was immunized followed by a single boost with  $1 \times 10^4$  *uis3*(-) sporozoites each. The animals were then monitored for the parasitemia by daily blood smears. All animals remained blood stage parasite-negative after the first immunization and subsequent boosts. Animals were challenged 7 days up to 1 month after receiving the last boost of *uis3*(-) sporozoites by intravenous or subcutaneous injection of either  $5 \times 10^4$  or  $1 \times 10^4$  infectious *P. berghei* WT sporozoites. For each set of experiments, at least three naive animals of the same age group were included to verify infectivity of the sporozoite challenge dose. In each naive animal, parasitemia was readily detectable at days three to five after injection by Giemsa-stained blood smears. Protected animals were monitored for at least 14 days and typically up to 1 month. A re-challenge study was performed for one immunization experiment two months after the first challenge with a single dose of  $5 \times 10^4$  infective *P. berghei* WT sporozoites. To test whether *uis3*(-) immunized mice were protected against re-challenge by natural transmission 10 protected and 5 naive control mice were exposed for 10 min to 10 highly infected mosquitoes that contained an average of 40,000 WT salivary gland sporozoites each. Successful blood-feeding was confirmed by mosquito dissection after the challenge experiment. To confirm stage-specificity of protection, an additional experiment was performed with 10 mice that were fully protected against a challenge with infective sporozoites. All immunized mice and three naive control mice were challenged by intravenous injection of  $5 \times 10^4$  *P. berghei* WT blood stage parasites. All mice were fully susceptible to blood stage inoculations with no differences in patency.

Results: Table 1 below shows that C57Bl/6 mice immunized with *P. berghei uis3(-)* sporozoites are completely protected against a challenge with WT *P. berghei* sporozoites.

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TABLE 1.

Exp.	Immunization #s <i>uis3(-)</i> spz.	Boosts:1st/2nd numbers (day)	Challenge dose (timepoint)	# Protected / # Challenged (pre-patency)
I.	50,000	25,000 (d.14)/ 25,000 (d.21)	10,000 spz. (d.7)	10 / 10 (no infection)
	10,000	10,000 (d.14)/ 10,000 (d.21)	10,000 spz. (d.7)	10 / 10 (no infection)
	—	—	10,000 spz.	0 / 9 (d.3)
II.	50,000	25,000 (d.34)/ 25,000 (d.45)	10,000 spz. (d.30)	5 / 5 (no infection)
	10,000	10,000 (d.34)/ 10,000 (d.45)	10,000 spz. (d.30)	5 / 5 (no infection)
	—	—	10,000 spz.	0 / 6 (d.4.5)
III.	50,000	50,000 (d.14)/ 10,000 (d.21)	10 inf. mosq. (d.38)	5 / 5 (no infection)
	10,000	10,000 (d.14)/ 10,000 (d.21)	10 inf. mosq. (d.38)	5 / 5 (no infection)
	—	—	10 inf. mosq.	0 / 5 (d.3)
IV	10,000	10,000 (d.14)/—	10,000 spz. (d.7)	7 / 10 (d.8)
	—	—	10,000 spz.	0 / 5 (d.3)
V.	50,000	25,000 (d.14)/ 25,000 (d.21)	10,000 blood st. (d.30)	0 / 5 (d.2)
	10,000	10,000 (d.14)/ 10,000 (d.21)	10,000 blood st. (d.30)	0 / 5 (d.2)
	—	—	10,000 blood st.	0 / 3 (d.2)
VVI.	50,000 s.c.	25,000 (d.11) s.c./ 25,000 (d.18) s.c.	10,000 spz. (d.23)	5 / 5 (no infection)
	50,000 s.c.	25,000 (d.11) s.c./ 25,000 (d.18) s.c.	50,000 spz. (d.23)	5 / 5 (no infection)
	—	—	10,000 spz.	0 / 6 (d.4.5)

Notes: Mice were immunized with *P. berghei uis3(-)* sporozoites. Mice were challenged with infectious *P. berghei* WT sporozoites or blood stages. Mice were from the same age group (50-80 days old) and sporozoites were from the same mosquito batch. Timepoints in column 4 indicate the day of challenge after the final boost. The pre-patent period is defined as the time until the first appearance of a single erythrocytic stage in Giemsa-stained blood smears. Five mice of the Exp. I. group were re-challenged with

10

one dose of 50,000 WT sporozoites 2 months after the first challenge and remained protected.

## EXAMPLE 2

Here, we disrupt another *Plasmodium* protein with a critical function for complete liver stage development. *UIS4* (upregulated in infective sporozoites gene 4) is expressed exclusively in infective sporozoites and developing liver stages. Targeted gene disruption of *UIS4* in the rodent model malaria parasite *Plasmodium berghei* generated knockout parasites that complete the malaria life cycle until after hepatocyte invasion. *UIS4* knockout parasites transform into early liver stages. However, they are severely impaired in further liver stage development and can only initiate blood stage infections when inoculated at high sporozoite doses. Immunization with *UIS4* knockout sporozoites completely protects mice against subsequent infectious wildtype sporozoite challenge. After sporozoite invasion of hepatocytes, *UIS4* localizes to the newly formed parasitophorous vacuole membrane that constitutes the parasite-host cell interface and extends as a tubo-vesicular network into the hepatocyte cytoplasm. Together our data demonstrate that depletion of *UIS4* results in attenuated liver stage parasites. Genetically attenuated liver stages may induce immune responses, which inhibit subsequent infection of the liver with wildtype parasites.

Generation of *uis4*(-) parasites. Given that *UIS4* is expressed in sporozoites but not in blood stages, we were able to pursue a targeted gene disruption at the blood stages to study the importance of *UIS4* for the *Plasmodium* pre-erythrocytic life cycle stages. The endogenous *PbUIS4* gene was disrupted using the above-described insertion and replacement strategies (data not shown). The parental blood stage population from the successful transfection was used for selection of clonal parasite lines carrying the gene disruption. We obtained insertion/disruption clones designated *uis4*(-) and replacement clones designated *uis4REP*(-) that contained exclusively the predicted mutant locus. The correct replacement event was confirmed by insertion-specific PCR (data not shown). To confirm *PbUIS4* deficiency of the mutant parasites we performed RT-PCR and cDNA

amplification using polyA<sup>+</sup> RNA from salivary gland sporozoites as templates (data not shown). Moreover, Western blot analysis of *uis4REP*(-) sporozoites did not detect PbUIS4 (data not shown).

*Plasmodium berghei* transfection and genotypic analysis. For gene targeting of  
 5 *PbUIS4* a 582 bp fragment was amplified using primers UIS4INTfor  
 (5' CGGAATTCATCATATTACTAATTTTCGGGGG 3') (SEQ ID NO: 5) and  
 UIS4INTrev (5' TCCCCGCGGTTATTCCATGTTATAAACGTTATTTCC 3') (SEQ ID  
 NO: 6) using *P. berghei* genomic DNA as template. Cloning into the *P. berghei* targeting  
 vector (13) resulted in plasmid pAKM15. Parasite transformation and selection was  
 10 performed as described previously (13). Integration-specific PCR amplification of the  
*uis4*(-) locus was achieved using the following primers: test1, *T. gondii* DHFR-TS for  
 (5' CCCGCACGGACGAATCCAGATGG 3') (SEQ ID NO: 7) and *UIS4* test rev  
 (5' CCCAAGCTTAGTTTGCATATACGGCTGCTTCC 3') (SEQ ID NO: 8); test 2, *UIS4*  
 test for (5' CGGAATTCTGGATTCATTTTTTTGATGCATGC 3') (SEQ ID NO: 9) and T7  
 15 (5' GTAATACGACTCACTATAGGC 3') (SEQ ID NO: 10). For replacement of *PbUIS4*  
 two fragments 1 kb and 600 bp were amplified using primers UIS4rep1for  
 (5' GAATTCTGGATTCATTTTTTTGATGCATGC 3') (SEQ ID NO: 11) and  
 UIS4rep2rev (5' GGGGTACCTTTATTCAGACGTAATAATTATGTGC 3') (SEQ ID  
 NO: 12) for the 1kb fragment and UIS4rep3for  
 20 (5' AAAACTGCAGATAATTCATTATGAGTAGTGTAATTCAG 3') (SEQ ID NO:13)  
 and UIS4rep4rev (5' CCCCAAGCTTAAGTTTGCATATACGGCTGCTTCC 3') (SEQ  
 ID NO:14) for the 600 bp fragment using *P. berghei* genomic DNA as template. Cloning  
 into the *hDHFR* targeting vector (34) resulted in plasmid pAKM17. To detect *UIS4*  
 expression in WT and mutant *P. berghei* parasites, 1 x 10<sup>5</sup> salivary gland sporozoites  
 25 were dissolved in 10 microliters SDS sample buffer. UIS4 was visualized on Western  
 blots using the polyclonal UIS4 antisera (12) and horseradish peroxidase-coupled anti-  
 rabbit IgG secondary antibody (Amersham). For RT-PCR analysis we dissected 8 x 10<sup>5</sup>  
*uis4*(-), 8 x 10<sup>5</sup> *uis4REP*(-) and 4 x 10<sup>5</sup> WT salivary gland sporozoites and isolated



polyA<sup>+</sup> RNA using oligo dT-columns (Invitrogen). For cDNA synthesis and amplification we performed a two step-PCR using random decamer primers (Ambion) and subsequent standard PCR reactions.

Phenotypic analysis of *uis4(-)* parasites. *Anopheles stephensi* mosquitoes were raised under a 14 h light/10 h dark cycle at 28°C, 75% humidity and were fed on 10% sucrose solution. Blood-feeding and mosquito dissection was as described (35). The number of sporozoites per infected mosquito was determined in a hemocytometer. To analyze sporozoite motility, sporozoites were deposited onto precoated glass coverslips and incubated using primary antibody against *P. berghei* circumsporozoite protein (anti-PbCSP) (35). Bound antibody was detected using Alexa Fluor 488-conjugated anti-mouse antibody (Molecular Probes). To detect liver stages in hepatocytes, *P. berghei* sporozoites were added to subconfluent hepatocytes, incubated 2 h at 37°C, and washed off. After 12, 24, 36 and 48 h, liver stages were revealed using primary antibodies against parasite heat shock protein 70 (HSP70) and a secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes). To analyze sporozoite invasion, 3 x 10<sup>4</sup> salivary gland sporozoites were added to subconfluent HepG2 cells and incubated for 90 min at 37°C. The ratio between intracellular and extracellular parasites was visualized using a double staining protocol with the anti-CSP antibody (36) and confocal microscopy. To determine the infectivity of clonal sporozoite populations *in vivo*, C57/Bl6 mice were injected intravenously or subcutaneously with 100 microliters sporozoite suspension of WT parasites or knockout parasites in RPMI 1640. Parasitemia of the animals was checked daily by examination of a Giemsa-stained blood smear. The appearance of a single erythrocytic stage represents the first day of patency.

Immunization and parasite challenge experiments. For all experiments female C57BL/6 mice (Charles River Laboratories) aged between 50 and 80 days were used. For immunizations, *uis4REP(-)* sporozoites were extracted from the salivary glands from infected mosquitoes. Sporozoites were injected in a volume of 100 microliters intravenously into the tail vein of the animals. Animals were immunized with a single

dose of 10,000 or 50,000 *uis4REP*(-) sporozoites, followed by two boosts of either 10,000 or 25,000 *uis4REP*(-) sporozoites administered i.v. The first boost was given 14 days following the immunization, with a second boost following 14 days thereafter. The animals were then monitored for parasitemia by daily blood smears. Only those animals that remained blood stage parasite-negative after the first immunization and subsequent boosts were exposed to a challenge with WT sporozoites. Animals were challenged 10 days after receiving the last boost of *uis4REP*(-) sporozoites by intravenous injection. All challenges consisted of 50,000 infective *P. berghei* WT sporozoites. For both sets of experiments, 5 naive animals were included to verify infectivity of the sporozoite challenge dose. In each naive animal, parasitemia was readily detectable at day 3 after injection. Starting from day 3 after WT challenge, the *uis4REP*(-) sporozoite-immunized animals were examined for detectable parasitemia in Giemsa-stained blood smears. Animals did not show a detectable parasitemia within 50 days following the challenge and were considered completely protected.

Results are shown in Table 2 below. Immunization with *uis4REP*(-) sporozoites confers sterile protection. The fact that a large proportion of mice remained blood stage negative after inoculation with *uis4REP*(-) sporozoites allowed us to test if immunization with these attenuated sporozoites would protect mice against WT sporozoite challenge. Therefore, we immunized C57/bl6 mice with 3 doses of 50,000 or 10,000 *uis4REP*(-) sporozoites and subsequently challenged the mice, which remained blood stage negative after immunization, with 50,000 infectious WT sporozoites (Table 2). None of the immunized mice developed blood stage infections after challenge and therefore enjoyed complete, sterile protection. Naive mice that were challenged with 50,000 WT sporozoites developed blood stage infections 3 days after inoculation.

Table 2. C57Bl/6 mice immunized with *uis4REP*(-) sporozoites are completely protected against a challenge with WT sporozoites.

TABLE 2.

Immunization ( <i>uis4REP</i> (-) spz.)	Boosts (days after immun./# of spz.)	# Protected/ # Challenged (prepatency)
50,000	1 <sup>st</sup> (14 / 25,000), 2 <sup>nd</sup> (28 / 25,000)	8 / 8 (no infection) <sup>1</sup>
none	none	0 / 5 (day 3) <sup>2</sup>
10,000	1 <sup>st</sup> (14 / 10,000), 2 <sup>nd</sup> (28 / 10,000)	8 / 8 (no infection) <sup>1</sup>
none	none	0 / 5 (day 3) <sup>2</sup>

Notes: <sup>1</sup>Immunized mice were challenged with 50,000 WT *P. berghei* sporozoites at day 38 after immunization. Mice were from the same age group and sporozoites were from the same mosquito batch. Blood smears were evaluated up to day 50 after challenge. <sup>2</sup>Naive control mice were from the same age group and challenged with 50,000 WT *P. berghei* sporozoites.

Our findings demonstrate that malaria parasites harbor genes that are necessary only for successful completion of the pre-erythrocytic mammalian infection, within hepatocytes. We have shown that deletion of such a gene effectively creates genetically attenuated malaria parasites that infect the liver of the mammalian host but are severely impaired in their ability to further progress through the life cycle and cause malaria disease. Other genes in the *Plasmodium* genome, which are critical for liver stage development, can be identified with the materials and methods described herein.

Finally, we have shown here that immunization with UIS4 knockout sporozoites confers complete, sterile protection against subsequent infectious sporozoite challenge in a mouse model. This demonstrates the successful use of genetically attenuated *Plasmodium* parasites as live experimental vaccines. Genetically attenuated human *Plasmodium* parasites may be similarly prepared as whole organism vaccines against malaria.

### EXAMPLE 3

This third example describes a representative protocol for making a *UIS3*-like knockout in *P. falciparum*.

The *P. falciparum* *UIS3* gene is targeted for disruption by replacement via a well-known double-crossover recombination strategy. The *UIS3* locus is replaced by a fragment containing the 5' and 3' untranslated regions of the *P. falciparum* *UIS3* open reading frame, each flanking the human dihydrofolate reductase (*dhfr*) selectable marker. Sequence data for the *P. falciparum* *UIS3* locus were obtained from the PlasmoDB database (www.plasmodb.org). The accession number for the coding sequence of *P. falciparum* *UIS3* is PF13\_0012 (12) and the location of the exon within chromosome 13 is 123930 –124619 on the minus strand. The *P. falciparum* *UIS3* rep1 fragment extends from nucleotides 124609-125594, and the rep2 fragment from 122872-123921.

PfUIS3 rep 1 and 2 fragments are amplified from *P. falciparum* 3D7 genomic DNA using Expand polymerase and the following primers: PfUIS3 rep1 forward 5'-GAGTAATATAATGTGTAATGCATATGG-3' (SEQ ID NO:15) and reverse 5'-GAGACCTTCATTTCAAAAAGGAAG-3' (SEQ ID NO:16); PfUIS3 rep2 forward 5'-CAAATGAAAACCTTGGAATAATCAGACGAG-3' (SEQ ID NO:17) and reverse 5'-GTATTATGCTTAAATTGGAAAAAAGTTTGAAG-3' (SEQ ID NO:18). The sizes of the rep1 and rep2 fragments amplified are 986 and 1051 base pairs, respectively. The PCR conditions are: one cycle of 94°C for 3 min, followed by thirty cycles of 94°C for 30 sec, 54.5°C for 1 min, and 65°C for 3 min.

The PCR products are digested and cloned into the pHTK (47) vector. Rep1 was cloned into restriction sites BglII and SacII, and rep2 into EcoI and SfoI sites. The PfUIS3 replacement construct is sequenced to confirm correct cloning. Positive selection for transfected parasites carrying the *dhfr* gene is carried out with the drug WR99210. pHTK contains the gene for thymidine kinase, allowing for negative selection of parasites carrying the plasmid episomally.

A similar protocol may be used for making a knockout of any gene of interest in *P. falciparum* (for example, a *UIS4*-like gene, accession number NP\_700638, PF10\_0164), or for making a knockout of such genes in other *Plasmodium* organisms.

Genomic information, including genomic sequences, ESTs, annotations, automated predictions, SAGE tags, microarray data, mapping data, and open reading frames, for many Plasmodium organisms, including, for example, *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. yoelii*, *P. chabaudi*, *P. reichenowi*, and *P. gallinaceum*, is readily available in  
5 public databases such as the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), the Plasmodium Genome Database ([www.plasmodb.org](http://www.plasmodb.org)), and the Sanger Institute ([www.sanger.ac.uk](http://www.sanger.ac.uk)).

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20 While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.